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A Genome-Wide Knockout Screen to Identify Genes Involved in Acquired Carboplatin Resistance

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14. ABSTRACT

The specific aims of this project are to: 1) Perform a GeCKO library screen to identify genes that when knocked out render human ovarian cells > 2.5-fold resistant to CBDCA; 2) Validate the ability of candidate gens to control sensitivity to CBCDA *in vitro* and *in vivo*, and 3) Functionally annotate the candidate genes using public resources. Substantial progress has been made on specific aim 1. This progress report covers work done from June 15, 2015 through May 31, 2016. Significant achievements to date include: the generation of three Cas9- expressing ovarian cell lines; the generation of CBDCA cytotoxicity killing curves in three ovarian cell lines; the production of GeCKO v2.0 lentivirus; and, tittering of virus against HEK293 cells and a panel of human ovarian carcinoma cell lines.

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INTRODUCTION

Specific Aims

The specific aims of this project are to: 1**)** perform a GeCKOv2 library screen to identify genes that when knocked out render human ovarian cells > 2.5-fold resistant to CBDCA; 2) validate the ability of candidate gens to control sensitivity to CBCDA *in vitro* and *in vivo*; and, 3) functionally annotate the candidate genes using public resources. Substantial progress has been made on specific aim 1. This progress report covers work done during months the first 11 months of the grant (June 15, 2015 – May 1, 2016).

Cisplatin/carboplatin resistance

Carboplatin (CBDCA) in combination with paclitaxel is the standard first line therapy for ovarian cancer patients and while initial response rates are high, almost all patients relapse and eventually become resistant to further treatment with Pt-containing drugs. One of the disappointments of the past decade is the pharmacologic, cell biological and expression profiling approaches have failed to clearly identify the molecular basis for resistance. CBDCA can induce mutations in DNA and there is very high probability that resistance is primarily due to point mutations or indels that disable the function of either regulatory or coding regions such that the expression or structure of one or more proteins is altered. Virtually no information is available on either the frequency of such mutations or the number of genes that might be targets. The CRISPR-cas9 technology now permits the facile somatic knockout of both copies of a gene using a guide RNA (gRNA) and the cas9 endonuclease [reviewed in (1)].

CBDCA and cisplatin (cDDP) produce the similar adducts in DNA. While the majority of these lesions are removed by the nucleotide excision repair process, some persist and are the source of mutations. A single exposure to cDDP generates clones in the surviving population that are highly resistant not only to cDDP and CBDCA but also to most drugs used clinically in combination with either cisplatin or CBDCA (2). Ovarian cancer cells selected for acquired CBDCA resistance have the following characteristics: 1) they are cross resistant to cisplatin (3); 2) the resistance is quite stable over many cell generations; 3) the phenotype is dominant as demonstrated by the fact that fusions between sensitive and resistant cells are resistant (4); and, 4) it is almost always associated with reduced drug accumulation (5-13). Our expression profiling of 6 isogenic pairs of human ovarian cancer cell lines, each consisting of a cDDP/CBDCA-sensitive parental line and a subline selected for acquired resistance, disclosed statistically significant differential expression of hundreds to several thousand genes in each pair, but did not disclose any one gene or set of genes that were consistently altered in all 6 pairs (14). Other expression profiling and systems biology approaches based on expression profiling have yielded similar disappointing results; in all probability this failure is due to the difficultly of teasing out a clear signal from quite noisy small datasets (15,16). There is ample evidence that defects in several different DNA repair pathways can result in hypersensitivity to CBDCA and cDDP, but it remains uncertain whether increased DNA repair capacity is the basis for resistance (17). That the loss of a single gene can result in resistance is demonstrated by the fact that mutations in the DNA mismatch repair genes MLH1 or MSH2 produce resistance due to failure of the cell to detect and process the DNA adducts correctly (18), but there is no information as to whether this mutation is a common cause of acquired resistance in either cell lines or clinical samples. The CRIPSR-cas9 technology now provides us with a major new tool to introduce knock out mutations genome-wide and help us address this challenge.

Hypothesis

It is our **hypothesis** that one source of acquired CBDCA resistance is mutations in the coding sequence of genes that either stop protein production (stop mutations) or alter protein structure so as to disable function, and that these genes and the pathways in which they function can be identified using a genome-wide knockout screen with the GeCKO library.

BODY

The focus of the work during the first 11 months of this project has been on Specific Aim #1 which is to perform a genome wide functional genomic screen using a CRISPR library to identify genes that when knocked out render human ovarian cells > 2.5-fold resistant to CBDCA.

Library selection

Since the application for this grant was originally written several lentiviral libraries of guide RNAs targeting most human genes co-expressed with cas9 from a lentiviral vector have become commercially available from Addgene in addition to the original GeCKO library. Among these we opted to work with the GeCKOv2 library. This library has already been used in both positive and negative screens to identify genes of interest.
The GeCKOv2 lentiviral library was produced in the laboratory of Dr. Feng Zhang at MIT. Each retrovirus in the library expresses both Cas9 and a single guide RNA (sgRNA). The library contains viruses expressing 3-4 sgRNA for each of the 18,080 target genes for a total of ~64,000 sgRNAs. In constructing the library the authors took extra care to prevent off target editing. They selected the sgRNA sequences with the lowest off target score, based on a normalized distance and location of matched nucleotides. They also established the screening conditions, including optimizing the MOI, to ensure unbiased representation during the infection. They further verified that the genome modification increases overtime to reach near completion 7 days after infection. The off-target effect was check by sequencing up to 5 predicted off target loci for a few sgRNA and they demonstrated that off-target cutting was found in only a very small fraction of the cells but that it increases with time post infection. In collaboration with other laboratories at UCSD, the GeCKOv2 library was obtained from Addgene and is currently being used for several different types of screens.

CRIPSR working group

Multiple laboratories at UCSD are now using the CRISPR-Cas9 technology to edit the genome. To facilitate this research, we established a working group that involves major leaders in the development of CRISPR (Prashant Mali, Ph.D., Gene Yeo, Ph.D.) and informatics (Trey Ideker, Ph.D.) technology. We have also established a listserve to facilitate information exchange between laboratories (crispr-screens-l@mailman.ucsd.edu).

Determination of the lentivirus infectability of ovarian cancer cell lines

Some cell lines like HEK293T are easily infected by lentivirus. In contrast, ovarian cancer cell lines are more difficult to infect in general. We first tested the infectability of 5 ovarian cancer cell lines, COV318, CAVO3, UCI-107, OVCAR-8 and Kuramochi using LV-SIN-CMV-EGFP virus and compared the fraction of GFP-positive cells to obtained with the HEK293T cell line. With this batch of virus flow cytometric results demonstrated that 91.5 % of HEK293T cells were infected and expressed GFP. CAOV3 is the most easily infected of the ovarian cancer cells with 97.3 % of cells becoming GFP-positive, followed by COV318 (62.9 %), UCI-107 (25.4 %), and OVCAR-8 (16.3 %). On the basis of GFP expression only 14 % of the Kuramochi cells became infected. We have previously used CRISPR-Cas9 genome editing to individually knock out several human copper homeostatic proteins in OVCAR-8 cells (19). Therefore, CAOV3, UCI-107 and OVCAR-8 were selected for the transduction with a Cas-9-expressing vector to create a population of cells

that expressed high levels of the enzyme. Although it seemed COV318 is more easily infected by LV-SIN-CMV-EGFP virus than UCI-107, it grows very slowly with doubling time of 72 h which is not ideal for the single clone isolation and later GeCKOv2 library screening.

Generation of Cas9-expressing ovarian cell lines

LentiCas9-blast is a lentiviral construct that expresses hSpCas9 and a blasticidin resistance gene. We used this vector to introduce Cas9 into each of the 3 ovarian cancer cell lines. OVCAR-8, CAOV3 and UCI-107 were transduced with lentiCas9-blast virus and then selected with 10 µg/mL blasticidin for 10-14 days. To ensure consistent, high level Cas9 expression in a uniform genetic background, at least 10 single clones were isolated for each cell line and the expression levels of Cas9 were measured by western blot analysis. Figure 1 is a representative Western bot of the heterogeneous expression of Cas9 from each of a set of single clones isolated from OVCAR-8 cells, emphasizing the importance of single clone isolation to ensure the equal Cas9 editing efficiency for the library screening.

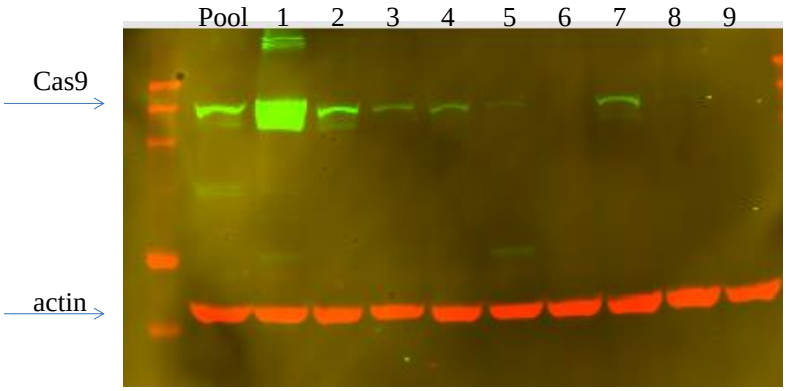

Figure 1. Cas9 expression levels in OVCAR-8-Cas9-pooled population and single clones 1-9

Determination of optimal CBDCA concentrations for use in ovarian cancer cell line screens

On theoretical grounds, and based on the experience of others, we determined that we want an exposure for CBDCA that kills approximately 50% of the cells during the first 7 days after the start of drug exposure but allows recovery of population growth by 14 days. In order to mimic the clinical use of CBDCA with its relatively short initial half-life we opted to use a 1 h exposure rather than a more prolonged or continuous exposure. CAVO3, UCI-107 and OVCAR-8 cells were exposed to various concentrations of CBDCA for 1 h following which the cells were washed with PBS three times and cultured in drug-free media for remainder of the experiment. Cell numbers were counted every 3-4 days to document the recovery of cells from CBDCA treatment (Figure 2). We found that CAOV3 cells are intrinsically quite a bit more resistant than OVCAR-8 and UCI-107. We selected 400 µM, 800 µM and 1.5 mM CBDCA for OVCAR-8, UCI-107 and CAOV3 cells, respectively, for genome-wide functional screening with the GeCKOv2 library with the goal of identifying genes that mediate resistance to CBDCA.

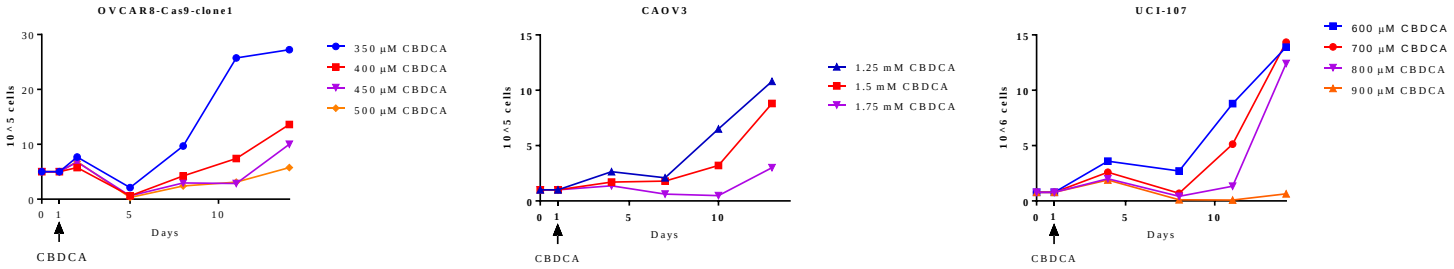

Figure 2. Cell counts of OVCAR-8-Cas9-clone 1, CAOV3 and cells after treated with various concentrations of CBDCA.

Production of GeCKO plasmid library and packaging into virus

The GeCKOv2 library consists of over 120,000 unique CRISPR gRNAs, six per gene. Because the plasmid library can be amplified in bacteria and re-extracted, the GeCKO library is a renewable resource; however, due to the large number of unique elements care must be taken to avoid loss of library complexity. Working with collaborators in the laboratory of Trey Ideker, Professor of Medicine and Bioengineering at UCSD and member of the Moores Cancer Center, we have optimized protocols to expand the GeCKOv2 plasmid library in bacteria, re-extract the library using a commercial column-based kit from Zymo Research (Irvine, CA), and then package this plasmid into high titer lentivirus. Complexity of the library, that is the relative distribution of reads per individual gRNA in the pool, was measured before and after amplification by performing Next-Generation Sequencing (NGS) of the gRNA regions and then sequencing on an Illumina MiSeq sequencer. The amplification process did not reduce library complexity as seen by similar shape of distribution curves before (Addgene) and after amplification and extraction (Figure 3). Quantitatively, although in this sequencing run there were more total reads for the Addgene sample, there was no significant difference in the fraction of gRNA with at least one read (98.7% vs. 96.8%) and the differential representation ratio between the 90th and 10th percentile of gRNA was also similar (7.0 vs. 7.3). For reference, the quality control cut offs used by the Zhang group were > 90% representation and < 15-fold ratio between 90th and 10th percentile. After production of more plasmid library DNA the GeCKO library must be packaged into lentivirus. We have now optimized viral production in 293T HEK cells. Using Centricon spin filters from Millipore (Billerica, MA) to concentrate virus we now routinely achieve titer in range of 10^8 pfu/mL based on titer experiments in HEK-293T cells.

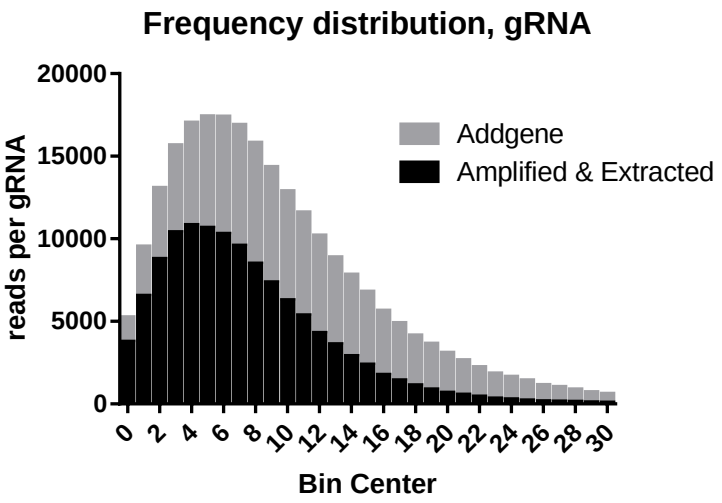

Figure 3. Frequency distribution of gRNA before and after amplification.

GeCKO v2 lentiguide-puro viral functional titration

With the assistance of the Gene Transfer and Targeting core laboratory at the Salk Institute tested the lentiviral preparation on HEK-293T cells and used primers which span the U5-Gag interface, a region common to all lentiviral preps, to measure the integration into the host cell DNA. The titer of our GeCKO v2 virus was 1.54 x 10^9 TU/mL. We measured the functional titer of our virus in OVCAR-8-Cas9-1 cells. Two hundred

thousand OVCAR-8-Cas9-1 cells were infected with 1.5 x 10^6 TU GeCKO v2 viral particles for 24 h, cultured in regular media for another 24 h and then selected with puromycin for 4 days. Only 6 x10^5 cells survived puromycin selection which was only 30 % of the uninfected control cells not treated with puromycin. The functional titer of the GeCKO v2 lentiguide-puro virus in OVCAR-8-Cas9-1 cells was only about 10% of that in the HEK-293T cells which was similar to the result of LV-SIN-CMV-EGFP viral transduction. We are currently determining the functional titer of GeCKOv2 lentiguide-puro virus in the CAOV3 and UCI-107 cells.

KEY RESEARCH ACCOMPLISHMENTS

- Identified optimal gudie RNA library
- Established CRIPSR working group
- Determined the lentivirus infectability of a panel of ovarian cancer cell lines
- Engineered ovarian cancer cell lines to express high levels of Cas9
- Determined optimal CBDCA concentrations for use in ovarian cancer cell line screens
- Produced GeCKO plasmid library and packaged into virus
- Titered the GeCKO v2 lentiguide-puro library against a panel of ovarian cancer cell lines

REPORTABLE OUTCOMES

Presentations

None

Abstracts

None

CONCLUSIONS

We have completed all of the preparative steps needed for the CRISPR-based screen for genes that when knocked out result in resistant to CBDCA. We anticipate completing screens in each line within the next two months.

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